Novel relaxant effects of RPL554 on guinea-pig tracheal smooth muscle contractility

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Background and Purpose.

We investigated the effectiveness of RPL554 a dual phosphodiesterase 3 and 4 enzyme inhibitor, on airway smooth muscle relaxation and compared it to that induced by salbutamol, ipratropium bromide, glycopyrrolate or their combination on bronchomotor tone induced by different spasmogenic agents.

Experimental Approach.

Guinea-pig tracheal preparations were suspended under 1 g tension in Krebs Henseleit solution maintained at 37^{o}C and aerated with 95% O_2/5% CO_2 and incubated in the presence of indomethacin (5 μM). Relaxation induced by cumulative concentrations of muscarinic receptor antagonists (ipratropium bromide or glycopyrrolate), beta2-agonists (salbutamol or formoterol), a PDE3 inhibitor (cilostamide, cilostazol or siguazodan) or a PDE4 inhibitor (roflumilast) was evaluated in comparison with RPL554. Maximal relaxation was calculated (% Emax papaverine) and expressed as mean ± sem.

Key Results.

Bronchomotor tone induced by the various spasmogens was reduced by the different bronchodilators to varying degrees. RPL554 (10-300 μM) caused near maximum relaxation irrespective of the spasmogen examined, whereas the efficacy of the other relaxant agents varied according to the contractile stimulus used. In further studies to evaluate potential synergistic interaction between bronchodilators, RPL554 proved superior to salbutamol when either was combined with muscarinic receptor antagonists.

Conclusions and implications.

RPL554 produces near maximal relaxation of highly contracted respiratory smooth muscle and provides additional relaxation compared with that produced by other clinically used

bronchodilator drugs. This suggests that RPL554 has the potential to produce additional beneficial bronchodilation over and above that of maximal clinical doses of standard bronchodilators in highly constricted airways of patients.

Abbreviations

CFTR: cystic fibrosis transmembrane receptor (CFTR)

COPD: chronic obstructive pulmonary disease

DMSO: dimethylsulphoxide

LABA: long acting beta agonist

LAMA: long acting muscarinic antagonist

MABA: muscarinic antagonist beta agonist

PDE: phosphodiesterase

RPL554: ([9,10-Dimethoxy-2(2,4,6-trimethylphenylimino)-3-(*N*-carbamoyl-2-aminoethyl)-3,4,6,7-tetrahydro-2*H*-pyrimido[6,1-*a*]isoquinolin-4-one]

Tables of Links

LIGANDS		
carbachol	**histamine**	**salbutamol**
cilostamide	**iberiotoxin**	
cilostazol	**ipratropium bromide**	
formoterol	**LTC4**	
glycopyrrolate	**roflumilast**	

TARGETS
PDE3A
PDE3B
PDE4A
PDE4B
PDE4D

These tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPAR/BPS guide to Pharmacology (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/2016 (Alexander *et al.*, 2015).

Introduction

Bronchodilators including beta2-agonists and muscarinic receptor antagonists are used in acute and maintenance therapy of respiratory conditions including asthma and COPD (Barnes, 2011; Cazzola *et al.*, 2012). The clinical effectiveness of these bronchodilators may be reduced with both increased disease severity and also during exacerbation of disease symptoms. Hence, there is a need to increase the magnitude of bronchodilation to obtain greater clinical benefit. One approach is the administration of long acting beta2-agonists and muscarinic receptor antagonists in combination (Tashkin & Ferguson, 2013; Spina, 2014). Similarly, the combination of ipratropium bromide with the short acting beta2-agonist, salbutamol is often used in the management of acute asthma, although the added benefit of the combination over salbutamol alone is questionable (FitzGerald *et al.*, 1997; Garrett *et al.*, 1997).

We have previously reported the clinical effectiveness and absence of significant adverse clinical effects such as nausea, emesis and cardiovascular liability of RPL554 the dual phosphodiesterase (PDE) 3 and 4 inhibitor in patients with asthma and COPD (Franciosi *et al.*, 2013). The increases in FEV_1 observed with this drug are likely a consequence of relaxation and functional antagonism of airway tone as demonstrated in guinea-pig isolated

trachea (Boswell-Smith *et al.*, 2006) and human bronchial airways (Calzetta *et al.*, 2013; Calzetta *et al.*, 2015).

Sustained bronchoconstriction can lead to airway closure which is associated with an increase in disease severity, and risk of exacerbation (Gibbons *et al.*, 1996; Lee *et al.*, 2001). The use of inhaled bronchodilators during an acute exacerbation of disease can ameliorate the effect of increased smooth muscle tone on airway closure, but comparable or additional improvement in clinical indices of symptoms can also be achieved with concomitant treatment with intravenous theophylline (Ream *et al.*, 2001; Wheeler *et al.*, 2005) or the PDE3 selective inhibitor enoximone (Beute, 2014) additional to standard care with bronchodilators and glucocorticosteroids. Phosphodiesterase inhibitors, particularly dual acting inhibitors like RPL554, may also have greater functional antagonistic properties than standard inhaled bronchodilators that could provide additional benefit, particularly during an exacerbation of asthma.

We have hypothesised that RPL554 may offer additional bronchodilation in severely constricted airways and therefore could provide an adjunct to current treatment modalities in the clinic. In this study, we show that RPL554 produced a greater maximal relaxation compared with standard bronchodilators administered alone or combined during different experimental conditions to increase bronchomotor tone. In addition RPL554 enhanced the relaxant effect caused by the combination of salbutamol and anti-cholinergic agents. Furthermore, this airway smooth muscle relaxant profile exhibited by RPL554 appears to be unique and not merely the sum of the addition of a selective PDE3 (e.g. cilostamide) and a selective PDE4 inhibitor (e.g. roflumilast).

Methodology

Group Sizes

Group sizes are specified in the tables and figures and represent the number of animals used. Exact group sizes are shown in the tables and figures. In some instances, group sizes appear unequal as variables were taken from different experiments to calculate the overall global mean potency and Emax value for a particular drug under the same experimental conditions. In one instance (Table 1), potency values for some drugs were calculated from N=3 and 4 and served to give a qualitative measure of drug potency and no formal statistical analysis was performed.

Randomization

There was no formal randomization of tracheal tissue to organ baths, but rather the chance allocation of tracheal preparations to organ baths on any experimental day. In a similar fashion there was no formal randomization of spasmolytic to tissue preparation from the same animal in view of the fact that multiple tracheal tissue preparations were obtained from the sample animal. The observational unit was the animal.

Blinding

It was not possible to blind the operator to each spasmolytic agent applied to each organ bath. The concentration ranges varied between spasmolytic agents hence making it impossible to blind to treatment. The variables measured were not of a subjective nature, rendered blinding in this case unnecessary.

Normalization

Relaxation data was expressed as the maximum relaxation response to papaverine (100 μM) which elicited a complete reversal of bronchomotor tone. Cumulative concentration response curves were fitted to a 3 parameter logistic equation for the determination of potency EC50 and maximum relaxation. For each spasmolytic agent, an estimate of EC50 and Emax was obtained for each animal and then the geometric mean (95% Confidence interval) and mean (SD) of these parameter estimates was calculated. It is well established that concentration conforms to a lognormal distribution.

Statistical Analysis

Unless otherwise specified, parameter estimates are presented as mean $\pm$ SD and the number of animals used is indicated in the table figure legends. Cumulative concentration-response curves were fitted to a 3 parameter logistic equation (GraphPad Prism version 5). Relaxant potency is expressed as geometric mean and 95% confidence interval. For the analysis of synergy, the interaction index (alpha) and additive response for each concentration pair was calculated following simultaneous solution of the intercept equation and the sigmoidal concentration-response relationship for each spasmolytic agent using SAS version 9.3. A single sample t-test was used to determine evidence of synergy by comparing alpha with the population mean of unity. Comparisons between mean values were deemed statistically significant when $P < 0.05$. A Benjamini-Hochberg multiple comparison test was used to adjust P values (InvivoStat version 3.1). We also undertook an additional statistical analysis of data presented in Table 1. The contractile response of each component spasmogen (as a % of the total response) was compared between bath (within group), agonist (within group in the instance when 3 different contractile agonists were used) and their interaction (bath x agonist) to assess regional trachea differences in contractile potency. The subject (i.e animal)

was a blocking factor to control for between animal variability. Data was analyzed using SAS version 9.3.

Validity of animal species or model selection

Tracheal tissue from guinea-pig was used in these studies. The guinea-pig is a suitable model to study the action of spasmolytic agonists. Bronchodilator drugs including beta2-adrenoceptor agonists and muscarinic receptor antagonists inhibit contraction in guinea-pig's and are clinically effective. We have previously shown that the dual phosphodiesterase 3 and 4 inhibitor, RPL554, is a functional antagonist in this species and we have gone on to demonstrate the clinical effectiveness of this drug in subjects with asthma and COPD, again highlighting the utility of this model in the context of understanding the pharmacology of respiratory drugs. Furthermore, like human airways, guinea-pig trachea responds to spasmogenic agents including histamine and LTC_4, which are spasmogenic agents used in this study.

Ethical Statement

Ethical approval for the work undertaken in this study was granted by local Animal Welfare Ethical Review Board, King's College London and permissible under a United Kingdom Home Office approved project licence (PPL70/7498).

Animals

Male Dunkin Hartley guinea-pigs (250-300 g, 6 - 8 weeks, Harland UK).

Housing and Husbandry

Animals were housed in groups of six within a Home office (United Kingdom) designated Biological Services unit with a 12 h night/day light cycle and access to food and water ad libitum. The cages contained soft bedding and environmental enrichment for welfare purposes and the animals were allowed to acclimatize to this environment for 5-7 days after arrival to the Unit before use.

Tissue preparation

Male Dunkin Hartley guinea-pigs (250-300 g) were humanely killed by cervical dislocation. Trachea and lungs were removed immediately and placed in ice-cold Krebs-Henseleit solution. The composition of the Krebs-Henseleit solution, which was gassed with 95% 0_2: 5% CO_2 and maintained at 37^0C, was (mM): NaCl 118.0, KCI 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, glucose 11 and $CaCl_2$ 2.5 in the absence or presence of Indomethacin (5 μM).

Following careful removal of adherent fat and connective tissue, the trachea was cut into rings (approximately 2 mm i.d and between 1-2 mm thickness). Either four or eight tracheal rings were taken from each animal depending on the experiment undertaken to one or two of our quadruple organ bath set-ups, respectively. In the case when eight preparations were used from the same animal, then two different experiments were conducted between the set of four tracheal preparations. There is no evidence of regional differences in relaxant or contractile potency along the trachea (Preuss *et al.*, 1998). Furthermore, randomizing tissue to organ bath would mitigate against any potential influence of regional variation in tissue sensitivity along the length of the trachea. The tissue preparations were then placed in water-jacketed organ baths at 37^oC containing Krebs- Henseleit solution and connected to FT03C

transducers. Tracheal preparations were equilibrated, under 1 g resting load for 40 min, before commencement of the experiment. Tension was measured using a Powerlab system (ADI, version 5 software). There was no formal randomization of tracheal tissue to organ baths and it was therefore a chance allocation of tracheal tissues to organ baths on any experimental day.

Experimental Procedures

Contractile responses

After a 30 to 40 min equilibration period, tissues were contracted with 0.1 and 1 μM carbachol to confirm tissue viability. Following a washout period of 30-40 min some tissues were contracted with increasing concentrations of carbachol in order to obtain an estimate of contractile potency (EC50). After the contractile response had plateaued, tissues were subjected once more to another washing routine and left for a further 30-40 min to equilibrate.

In other experiments, guinea-pig tracheal tissues were contracted with either carbachol (10 x EC50), or combinations of spasmogens added sequentially as follows: histamine (10 μM) and carbachol (10 μM) in the absence or presence of leukotriene $(LTC)_4$ (100 nM), the latter added first to the organ both. In other experiments, tracheal tissue was contracted with a combination of KCl (40 mM) and carbachol (10 μM) added sequentially in the absence or presence of LTC_4 (100 nM) applied first.

In experiments conducted with LTC_4, tissues were pre-incubated for 45 min with L-serine borate (40 mM) and L-cysteine (5mM) to prevent leukotriene metabolism. For

experiments conducted with KCL (40 mM), the Krebs-Henseleit solution was modified by reducing the NaCl concentration to 83 mM in order to maintain osmolarity. Tissues were incubated in this modified Krebs-Henseleit solution for a period of 30 to 40 min prior to administration of spasmogens.

Relaxation studies

Following stabilization of the contractile response to the different spasmogens and their combination, cumulative concentration-response curves to various spasmolytic agents were superimposed upon this response. These included beta2-agonists (salbutamol, formoterol); muscarinic receptor antagonists (ipratropium bromide, glycopyrrolate), PDE3 inhibitors (cilostamide, cilostazol, siguazodan), PDE4 inhibitors (roflumilast) and the dual PDE3/4 inhibitor, RPL554. In other experiments, RPL554 was added in a cumulative fashion in tissues after cumulative additions of beta2-agonist or muscarinic receptor antagonist to provide evidence of additional relaxation. In further experiments, combinations (ratio) of spasmolytic agents were used and included ipratropium bromide and salbutamol (1:5.7), to mimic the dose combination used clinically in nebulizers by subjects with asthma (McNamara *et al.*, 2012),glycopyrrolate and RPL554 (1:9, 1:24, 1:226), roflumilast and cilostamide (1:1), and siguazodan and cilostamide (1:1). This approach was used in order to determine additivity or synergy with such combinations of relaxant agonists. We also chose to administer increasing concentrations of a combination of PDE3 and PDE4 inhibitor in a 1:1 ratio, rather than in a sequential fashion (i.e. a single concentration of one inhibitor followed by increasing cumulative concentrations of another) to avoid confounding due to loss of tracheal tension to either inhibitor in a sequence when comparing relaxation responses

between combination of PDE inhibitors and RPL554. Maximum relaxation was achieved with papaverine.

Analysis of Synergy

We took the opportunity of analyzing the data collected for evidence of a synergistic interaction between different spasmolytic agents in some of the experiments undertaken with different experimental conditions, using a mathematical approach to describe a synergistic interaction between RPL554 and glycopyrrolate in human bronchial tissue (Calzetta *et al.*, 2013). To determine synergy for any combination of drugs, denoted *a* and *b* respectively, requires the simultaneous solution of the cumulative response relationship for both drugs i.e. *f*(A) and *f*(B) and the intercept equation described below:

$$f(A) = \frac{EmA}{\left(1 + \frac{EC50_A}{a}\right)}$$

$$f(B) = \frac{EmB}{\left(1 + \frac{EC50_B}{b}\right)}$$

Where *EmA* and *EmB* represent the maximum relaxation response to drug alone; $EC50_A$ and $EC50_B$ represent relaxant potency for either drug alone, and a, and b the concentration of each component in a drug combination, respectively. The intercept equation a/A + b/B = 1, defines the zero interaction relationship between dose combinations, where the denominator terms refer to the concentration of drug which *alone* stimulates an equi-effective response to the drug combination as defined by the numerator terms. The simultaneous solution of these equations yields the zero interaction (i.e. additive) concentration-response relationship. The observed effector response for each concentration pair is compared with the corresponding additive response (observed-additive). Furthermore, solving the intercept equation for each

concentration pair results in the calculation of the interaction index (alpha) and values of alpha <1 or =1 denote synergy or additivity, respectively. (Berenbaum, 1989; Lamarre & Tallarida, 2008; Tallarida, 2012).

Materials

Ipratropium bromide, salbutamol sulphate, carbachol chloride, histamine chloride, potassium chloride, glycopyrrolate and formoterol fumarate, were prepared in Krebs-Henseleit solution. CFTRinh172, iberiotoxin, RPL554, roflumilast, cilostazol, and cilostamide were prepared using dimethylsulphoxide (DMSO). The final concentration of DMSO in the organ bath was less than 0.5%. Leukotriene C_4 (LTC_4) stock solution was obtained in methanol/ammonium acetate buffer (65:35) ($8x10^{-3}$M). L-cysteine stock solution (1M) was prepared using Krebs-Henseleit solution. L-serine borate stock solution was prepared using L-serine (1M) and boric Acid (1M) dissolved in water and adjusted to pH 7.4 with 10 M NaOH. Papaverine hydrochloride was prepared in distilled water. Stock concentrations of drug combinations of glycopyrrolate and RPL554 (1:9, 1:24 and 1:226); cilostamide and roflumilast (1:1); and siguazodan and roflumilast (1:1) were prepared in DMSO and ipratropium bromide and salbutamol ('Combivent' 1:5.7) prepared in Krebs-Henseleit solution. A stock concentration (10 mM) consisted of 1.49 mM ipratropium bromide and 8.51 mM salbutamol, and serial dilutions were then prepared. Dilutions of the stock concentration of all drugs and their combinations where made in Krebs-Henseleit solution. Source of drugs were as follows: RPL554 ([9,10-Dimethoxy-2(2,4,6-trimethylphenylimino)-3-(*N*-carbamoyl-2-aminoethyl)-3,4,6,7-tetrahydro-2*H*-pyrimido[6,1-*a*]isoquinolin-4-one] (Verona pharma plc and synthesized by Onyx Scientific, UK); L-Cysteine, L-Serine, carbachol, glycopyrrolate, ipratropium bromide, DMSO, histamine, indomethacin and papaverine hydrochloride

(Sigma-Aldrich Company Ltd, UK); cilostamide, iberiotoxin (Cayman Chemical, USA); roflumilast (Biovision Inc, USA); siguazodan and CFTRinh172 (Tocris Bioscience, UK); cilostazol and salbutamol sulphate (LKT laboratories, USA); formoterol fumarate, LTC_4 (Santacruz Biotechnology, Germany).

Results

RPL554 functional antagonism studies

Four different spasmogenic conditions were employed to increase bronchomotor tone, namely, 1) combination of histamine (10 μM) and carbachol (10 μM); 2) the combination of LTC_4 (100 nM), histamine (10 μM) and carbachol (10 μM) (Fig 1); 3) the combination of KCL (40 mM) and carbachol (10 μM) (Fig2 A,C,E,G); and 4) the combination of LTC_4 (100 nM), KCL (40 mM) and carbachol (10 μM) (Fig 2B,D,F,H). The cumulative tension generated for each contractile agonist and the degree of contraction expressed as a percentage of the total contractile response for each condition is summarized in Table 1. There was no significant difference in the magnitude of the total contractile response induced by different combinations of these contractile agonists (Table 1). We also compared the contractile response (% Emax, Table 1) for each component in the combination to assess for regional variation in contractile activity along the trachea. There was no evidence of a significant difference in regional variation in tracheal contractile response to the spasmogens used in this study ($P > 0.05$, Table 1 footnote).

In each of these conditions, RPL554, ipratropium bromide, salbutamol and the combination of ipratropium bromide and salbutamol (1:5.7; 'Combivent') caused a

concentration dependent relaxation response. The degree of reversal was dependent upon the combination of spasmogens used to induce bronchomotor tone. RPL554 fully reversed tracheal tension irrespective of which spasmogenic condition was used (Table 2, Fig 1-2). As anticipated, there was a reduction in relaxant potency with RPL554 particularly in tissues contracted with potassium ions (Fig 2, Table 2), but nonetheless, full reversal of tone was always achieved. Similarly, whilst the relaxant activity of salbutamol, ipratropium bromide and their combination was reduced in tissues exposed to KCL (40 mM), yet full relaxation was achieved with the further addition of RPL554 (Fig 2, Table 2). The relaxant potency and maximum relaxation response (expressed as a percentage of reversal of papaverine Emax) for each spasmolytic agent is summarized in Table 2.

The contractile response of tracheal tissue to LTC_4, histamine and carbachol was not fully reversed by the long acting muscarinic receptor antagonist, glycopyrrolate (EC50; 2.9 (0.9 – 8.8) nM, n=6; Relaxation % Emax papaverine, mean, SD; 61 $\pm$ 10 %, Emax), however, the addition of RPL554 fully reverse bronchomotor tone (data not shown).

The effect of PDE3 and PDE4 selective inhibitors on bronchomotor tone

We compared the potency of a range of PDE inhibitors in tissue contracted with carbachol (10 x EC50). The PDE3 inhibitor, siguazodan and the PDE4 inhibitor, roflumilast had lower relaxant activity at the concentrations tested with the maximum concentrations employed being at least 10-100 x greater than predicted from their isolated enzyme inhibitory potency (Fig 3A). As anticipated bronchodilators like salbutamol sulphate and formoterol fumarate caused full relaxation, which was also observed with the PDE3 inhibitors, cilostamide and cilostazol as well as the dual inhibitor RPL554 (Fig 3, Table 3).

Bronchomotor tone induced by a combination of histamine (10 μM) and carbachol (10 μM) was not fully reversed by the beta2-agonists salbutamol and formoterol or the PDE3 selective inhibitors, or cilostamide (Fig 3B), yet under these same conditions the dual PDE inhibitor, RPL554 were able to cause near maximum relaxation. The PDE4 selective inhibitor, roflumilast, had weak relaxant activity under these assay conditions. Unlike RPL554, a combination of cilostamide and roflumilast (1:1) did not fully reverse bronchomotor tone under these conditions (Fig 3B). In additional experiments, cilostazol, a structurally related dihydroquinolinone , did not produce full relaxation (EC50, 95% CI: 9173 (962 – 87440) nM; % Emax papaverine (SD): 64 (17), N = 5) alone or in 1:1 combination with roflumilast (EC50 value was not estimable with confidence; Emax 39 (11), N = 5).

In tissues contracted with LTC4 (100 nM), histamine (10 μM) and carbachol (10 μM) both cilostamide alone and in a 1:1 combination with roflumilast (1:1) or the structurally unrelated PDE3 inhibitor, siguazodan either alone and in combination with roflumilast (1:1) also demonstrated lower relaxant activity than RPL554 alone in tissues contracted (Fig 3C,D respectively; Table 3). For completeness, cilostazol either alone (EC50, 95 % CI; 2919 (694 – 12460) nM; % Emax papaverine, SD; 66 (12), N = 6) or in combination with roflumilast in a 1:1 ratio (1890 (77 – 46400) nM, 52 (12), respectively, N = 6) did not completely reverse bronchomotor tone under these experimental conditions.

Interaction studies

We were afforded the opportunity of investigating whether the combination of different spasmolytic agents produced a synergistic relaxation response under the different contractile conditions employed. The relaxation response to ‘combivent’ (ipratropium bromide and salbutamol, 1:5.7) was analysed for any synergistic interaction between the two component

bronchodilators in tissues contracted with LTC4, histamine and carbachol (Fig 4A, B) and LTC4, KCL and carbachol (Fig 4C,D). The data presented in Fig 4A and 4D were re-drawn from the data presented in Fig 1. The zero interaction (additive) relationship for each concentration of 'combivent' was calculated as described in the methods (Fig 3B, 3D; blue line). There appears to be evidence of a greater relaxation response with the combination of ipratropium bromide and salbutamol as evidence by a leftward and downwards shift of the concentration-response curve to this combination compared with the additive response, indicative of a synergistic interaction. The magnitude of this difference between the observed response and the additive response was significant at concentrations greater than 30 nM (% relaxation, mean (SD); 25.4 (9) *, 22.6 (6.6) *, 25.2 (6.6) * and 23.6 (6.6), respectively, $P < 0.05$ unadjusted, $*P < 0.05$, Benjamini-Hochberg procedure). The interaction index (alpha) was inestimable because the relaxation response for the combination exceeded the maximum response to either drug alone. This synergistic interaction was absent in tissues contracted in the presence of KCL (Fig 3D).

We also evaluated the effect of a combination of RPL554 and glycopyrrolate under different contractile conditions and found evidence of synergy (Table 4), as assessed by alpha values less than unity, and greater relaxation response compared with the additive response (i.e. zero interaction effect), for selective concentration pairs of different combination ratios in tissues contracted and LTC_4 (100 nM), histamine (10 μM) and carbachol (10 μM) (Fig 5A,C,E) and with histamine (10 μM) and carbachol (10 μM) (Fig 5B, D, F). We also observed a synergistic interaction between glycopyrrolate and RPL554 in tissues exposed to high concentration of potassium ions (Table 4, Fig 6). Thus, irrespective of the contractile conditions employed, evidence of synergy between these two bronchodilators was observed.

Further studies

We evaluated the effect of selective inhibitors of CFTR and maxi-K calcium activated potassium channels on airway smooth muscle relaxation to agents that elevate intracellular cyclic AMP in view of the purported role these ion channels in this response. The relaxant potency (data not shown) to salbutamol or RPL554 was not significantly altered by a CFTR inhibitor, CFTRinh 172 (10 μM) or the calcium-activated potassium channel blocker, iberiotoxin (100 nM) (Fig 7).

Discussion

In this study, RPL554 produced maximal airway smooth muscle relaxation irrespective of the conditions used to induce contraction of guinea-pig isolated trachea. In addition RPL554 enhanced the airway relaxant effects of salbutamol, ipratropium bromide and their combination. If this translates to the clinical setting the data suggests that RPL554 could provide additional bronchodilator relief in severely constricted airways in patients who have already received standard care.

Clinical studies have demonstrated the ineffectiveness of oral or inhaled PDE4 inhibitors to act as acute bronchodilators at doses which inhibit indices of the inflammatory response or disease process (Grootendorst *et al.*, 2003; Engelstaetter *et al.*, 2005; Rabe *et al.*, 2005; Fabbri *et al.*, 2009; Singh *et al.*, 2010). In contrast, inhaled or intravenously administered PDE3 inhibitors have been demonstrated to cause bronchodilation in subjects with asthma (Fujimura *et al.*, 1997; Myou *et al.*, 2003). Furthermore, RPL554, a dual PDE 3 and 4 inhibitor produced bronchodilation in asthmatic and COPD patients (Franciosi *et al.*, 2013). These clinical studies point to the importance of PDE3 rather than PDE4 as the major isoform which controls the levels of intracellular cyclic AMP within airway smooth muscle

cells in humans. However, the expression of PDE3A in endobronchial biopsies was reduced by 2 fold in asthmatic subjects compared with healthy controls. The expression pattern for PDE4 in endobronchial biopsies from patients with asthma was not described in this study, possibly because the levels were not altered between the groups (Yick *et al.*, 2013). PDE gene expression has been measured in airway smooth muscle cells maintained in culture. The expression of PDE4D was increased and PDE3A unchanged in cultured airway smooth muscle obtained from subjects with asthma compared with healthy control subjects (Trian *et al.*, 2011; Lin *et al.*, 2015).

The PDE activity profile in homogenates of human airway tissue also confirms the presence of PDE3 and PDE4 activity, although differences in expression patterns have been reported by different laboratories (de Boer *et al.*, 1992; Cortijo *et al.*, 1993; Torphy *et al.*, 1993) which might be due to regional differences in the expression pattern of PDEs within the respiratory tract, although for PDE3 this seems unlikely in view of the relaxation response seen with a PDE3 inhibitor in human airways irrespective of anatomical location (de Boer *et al.*, 1992; Torphy *et al.*, 1993). PDE4 accounted for the majority of total PDE activity found in tracheal smooth muscle cells from non-asthmatic subjects (Billington *et al.*, 2008), whilst PDE3A and to a significantly lesser extent, PDE4D, was shown to be expressed in airway smooth muscle cells from intrapulmonary airways from non-asthmatic subjects (Trian *et al.*, 2011). Neverthless, the expression of PDE4D was increased in airway smooth muscle cells derived from subjects with asthma (Trian *et al.*, 2011). The inhibition of PDE4, but not PDE3, using subtype selective inhibitors (Trian *et al.*, 2011), or siRNA for PDE4D5 (Billington *et al.*, 2008) augmented cyclic AMP levels in response to beta2-adrenoceptor stimulation, possibly suggesting a major role for this splice variant which represents a fraction of the total PDE4 pool in human airway smooth muscle cells, in the control of cyclic AMP levels following acute stimulation of the beta2-adrenoceptor.

Functional studies confirm clinical observations that in general PDE4 inhibitors including rolipram and RP73401 produce modest relaxation of human isolated airways, whereas greater relaxant activity is observed with PDE3 inhibitors including siguazodan. However, these functional responses are dependent on the degree of bronchial or tracheal tone and the spasmogenic agent used to contract human airway tissue and therefore is context dependent. In general, as airway contractility is increased the relaxant activity of PDE4 inhibitors like rolipram is reduced to a greater extent than PDE3 inhibitors like siguazodan (Qian *et al.*, 1993; Rabe *et al.*, 1993; Torphy *et al.*, 1993; Naline *et al.*, 1996; Schmidt *et al.*, 2000). Furthermore, this context dependence might also explain why in some studies, both PDE4 inhibitors (Qian *et al.*, 1993; Naline *et al.*, 1996) and PDE3 inhibitors (Torphy *et al.*, 1993) augmented the relaxation response to isoprenaline.

Similar to these findings in man, tracheal tissue from the guinea-pig also expresses both PDE3 and PDE4 isoenzymes and the relaxation response to rolipram and the PDE3 inhibitor, CL-930, was biphasic and the first phase response was relatively modest (Harris *et al.*, 1989). The relaxation or functional antagonism exerted by PDE3 and PDE4 inhibitors was also dependent on the spasmogenic condition employed, but when combined gave a greater inhibitory effect than either inhibitor alone (Turner *et al.*, 1994; Underwood *et al.*, 1994; Bernareggi *et al.*, 1999). The nature of the biphasic response reported with rolipram is unclear, but newer PDE4 inhibitors including roflumilast and cilomilast also produced weak or modest reversal of tone in guinea-pig trachea (Bundschuh *et al.*, 2001; Kobayashi *et al.*, 2012). The role of PDE4 in airway smooth muscle might be dependent on the spasmogens used to contract tissues. For example, contractile responses induced by carbachol, but not serotonin, KCL and arginine vasopressin is significantly reduced in PDE4D gene ablated mice, or in tracheal preparations from wild type mice treated with rolipram, that was specifically dependent upon functional antagonism exerted by endogenously released PGE2

(Mehats *et al.*, 2003). Hence, we also evaluated the relaxant activity of RPL554 in tissues contracted with various combinations of spasmogens.

In view of the possibility that the contribution of different PDE isoforms for the regulation of cyclic AMP levels within airway smooth muscle may vary depending on disease status and to the degree of airway tone, it would seem advantageous to inhibit both PDE3 and PDE4 in airway smooth muscle and thereby provide more effective bronchodilation in most circumstances. In this study we have demonstrated that the relaxant activity of PDE3 and PDE4 selective inhibitors is dependent upon the contractile agonists used to induce tracheal airway smooth muscle contraction. The PDE3 selective inhibitors cilostamide and cilostazol, produced maximal relaxation in tissues contracted with carbachol (10x EC50), whereas cilostamide was less effective in tissues contracted with different combinations of spasmogenic agonists. In all conditions, the PDE4 inhibitor roflumilast only produced a modest relaxation. We did not use concentrations of roflumilast greater than 1 μM in view of the sub nanomolar affinity of this drug for PDE4 (Bundschuh *et al.*, 2001). The combination of a PDE3 and PDE4 inhibitor in a 1:1 ratio produce a comparable relaxation response compared with either inhibitor alone. However, RPL554 demonstrated greater relaxant activity under all spasmogenic conditions used highlighting a novel finding in that the relaxation induced by RPL554 is more than merely the combined inhibition of separate PDE3 and PDE4 subtype selective inhibitors. The greater relaxant activity of RPL554 might be due its greater inhibitory potency for PDE3 (human platelet PDE3, 0.4 nM) (Boswell-Smith *et al.*, 2006) compared with cilostamide (human recombinant PDE3A; 27 nM) (Sudo *et al.*, 2000); alternatively the combined inhibition of PDE3 and PDE4 by a single molecular structure may be more optimal than the combination of two separable isoform selective PDE inhibitors. Alternatively, RPL554 might have a more favourable pharmacodynamic profile within airway smooth muscle cells compared with cilostamide,

although this seems unlikely in view of the ability of cilostamide to fully reverse bronchomotor tone induced by carbachol (10x EC50). Whether RPL554 has additional properties other than inhibition of phosphodiesterase 3 and 4 is currently unknown, although we can rule out a role for calcium activated K channels and the cystic fibrosis transmembrane receptor (CFTR), given that selective inhibitors of these channels (Jones *et al.*, 1990; Norez *et al.*, 2014) did not significantly alter the relaxation response to RPL554.

We also compared the effect of RPL554 to induce spasmolysis when combined with the muscarinic receptor antagonist, ipratropium bromide, the beta2-adrenoceptor agonist, salbutamol alone, and in a 1:5.7 combination which has been used to mimic the effects of the clinically used "Combivent" preparation in whole animal studies (McNamara *et al.*, 2012). As anticipated, the relaxant activity of salbutamol and ipratropium bromide and their combination was dependent upon the mixture of different contractile agonists used to induce bronchomotor tone. In tissues which were exposed to KCL (40 mM), it is clear that the relaxant activity of these spasmolytics was reduced, but in all cases, additional inhibition of bronchomotor tone was achieved with RPL554. If these results are translated to patients, one might speculate that dual PDE inhibitors, like RPL554 could offer additional bronchodilator activity in severely constricted airways. This would be consistent, at least in part, with the clinically effective bronchodilation observed with RPL554 in subjects with asthma and COPD (Franciosi *et al.*, 2013). In an analogous scenario, the combination of a LABA and a LAMA appears to be superior than either component drug alone in terms of improvement in trough FEV1 (Buhl *et al.*, 2015), and the clinical effectiveness of a MABA (Wielders *et al.*, 2013) in patients with COPD, suggest that single molecule 'dual' inhibitors may be as effective, if not more effective than monocomponent drugs. We have previously reported that RPL554 can elevate intracellular levels of cyclic AMP in human neutrophils (Boswell-Smith *et al.*, 2006), and other studies have shown that combination of a PDE3 with PDE4

inhibitor was more effective than either inhibitor alone in suppressing the generation of inositol(1,4,5) triphosphate induced by histamine and carbachol, though more effective against the former (Challiss *et al.*, 1998). Hence, RPL554 offers the possibility of inhibiting PDE3 and PDE4 isoenzymes functionally more optimally than selective PDE 3 and 4 inhibitors used in combination.

We did not extensively investigate the intracellular mechanism by which RPL554 gives rise to this additional relaxation response under different spasmogenic conditions, although we can rule out an involvement of membrane hyperpolarization via opening of calcium activated potassium channels or CFTR. The reduction in relaxant activity observed with beta2-adrenoceptor stimulation in tissues contracted with potassium chloride (Janssen *et al.*, 2004) and this study, could be explained by differences in calcium signalling within airway smooth muscles by depolarization versus agonists of G-protein coupled receptors (Perez & Sanderson, 2005). In contrast, RPL554 might retain the capacity to elevate intracellular levels of cyclic AMP downstream of a PKC dependent impairment of beta2-adrenoceptor coupling and/or desensitization (Pitcher *et al.*, 1992).

Drugs used in combination have the potential to act in an additive or synergistic fashion and clinical evidence for synergism has been demonstrated elsewhere for combinations of anaesthetics or pain relieving medication (Montes *et al.*, 2000; Manyam *et al.*, 2006; Filitz *et al.*, 2008). The question of whether mechanistically distinct spasmolytics can act additively or synergistically has not been fully addressed using mathematical approaches that have been developed to investigate this phenomenon (Berenbaum, 1989; Lamarre & Tallarida, 2008; Tallarida, 2012). Previous studies have suggested a synergistic interaction between beta2-adrenoceptor agonists and muscarinic receptor antagonists, though not analysed rigorously using a mathematical approach of this phenomenon (Rossoni *et al.*, 2007; McNamara *et al.*, 2012; Smit *et al.*, 2014). We found some evidence of synergy for

various combinations of salbutamol and ipratropium bromide, and between RPL554 and glycopyrrolate, with the response for some concentration pairs greater than would be expected if there was an additive effect (zero interaction). The later observed consistent with a previous study in human bronchial airways (Calzetta *et al.*, 2015). Interestingly, this effect was lost for the combination salbutamol/ipratropium bromide but not RPL554/glycopyrrolate, in tissues contracted with KCL (40 mM), again leading to the speculation that RPL554 is bypassing impaired beta2-adrenoceptor signalling and in this respect highlights another novel finding with the ability of this dual PDE3 and PDE4 inhibitor to maintain synergism under conditions in which a synergistic action to beta2-adrenoceptor stimulation is lost. It has been proposed that synergy between the relaxant effects of indacaterol and glycopyrronium is mediated in part via the opening of calcium activated K channels (Kume *et al.*, 2014). Under the conditions employed in our study, the high concentration of potassium would render these channels inoperable and hence hyperpolarization and closure of voltage operated calcium channels would not occur. This would be expected to functionally antagonise the relaxation response to combination ipratropium bromide and salbutamol and hence, could explain this loss of synergy. However, in our studies iberiotoxin did not impair the functional response to salbutamol or RPL554, ruling out a role for these ion channels in the relaxation response to these cyclic AMP elevating drugs. The change in responsiveness to salbutamol was small compared with other reported studies using charybdotoxin or iberiotoxin and may be related to differences in the contractile stimulus employed in this study (Jones *et al.*, 1990; Corompt *et al.*, 1998). However, the importance of these ion channels in mediating relaxation of airway smooth muscle by beta2-adrenoceptor agents is not universally accepted (Huang *et al.*, 1993; Corompt *et al.*, 1998; Janssen, 2002; Janssen *et al.*, 2004; Sausbier *et al.*, 2007). Moreover, calcium channel blocking drugs do not appear to modify the relaxation to isoprenaline in isolated airway preparations (Janssen *et al.*, 2004).

In summary, in circumstances where the spasmolytic activity of a muscarinic antagonist or beta2-adrenoceptor agonist alone, or in combination is reduced, we have shown that, i) RPL554 provides additional smooth muscle relaxation when administered subsequently, ii) there was evidence of specific synergism between that RPL554 and glycopyrrolate, iii) that the relaxation produced by RPL554 was greater than that produced by the combination of a selective PDE3 and PDE4 inhibitor and iv) that the mechanism of airways smooth muscle relaxation is not due to activation of maxi-K channels or CFTR activation. This suggests that RPL554 could not only provide additional benefit to standard care in asthma and COPD, but under certain circumstances demonstrate a synergistic interaction for smooth muscle relaxation beyond that achieved with salbutamol. The additional anti-inflammatory properties described for RPL554 highlight its potential in the treatment of respiratory diseases.

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Conflict of Interest

No conflicts of interest.

Table 1. Summary of the contractile response to each spasmogen alone and in various combinations.

Contractile agents	**†Tension (mN)**	**††Contractile response (% Maximum)**
Carbachol (10 x EC50)		
Carbachol (10x EC50)	28 ± 7 (14)	100
Histamine/Carbachol		
Histamine (10 μM)	11 ± 7 (20)	32 ± 34 (20)
Carbachol (10 μM)	35 ± 21 (20)	100
LTC4/Histamine/Carbachol		
LTC4 (100 nM)	13 ± 6 (29)	47 ± 20 (29)
Histamine (10 μM)	21 ± 7 (29)	78 ± 13 (29)
Carbachol (10 μM)	27 ± 9 (29)	100
KCL/Carbachol		
KCL (40 mM)	13 ± 8 (10)	42 ± 21 (10)
Carbachol (10 μM)	31 ± 9 (10)	100
LTC4/KCL/Carbachol		
LTC4 (100 nM)	8 ± 8 (13)	26 ± 20 (13)
KCL (40 mM)	19 ± 9 (13)	71 ± 15 (13)
Carbachol (10 μM)	27 ± 8 (13)	100

Values expressed as mean ± SD with number of animals denoted in parenthesis.
† Cumulative tension generated by spasmogens. †† Contractile response to a spasmogen is expressed as a percentage of the total contractile response. We also compared the contractile response (% Emax) for each component in the combination to assess for regional variation in contractile activity along the trachea. Contractile response was analyzed by fitting to a model defined by fixed factors (organ bath) with the animal as a blocking factor. There was no significant bath effect in the case of Histamine/Carbachol and KCL/Carbachol, or interaction between tissue randomized to organ bath and the agonist used in the combination LTC4/histamine/carbachol and LTC4/KCL/Carbachol ($P > 0.05$), highlighting a lack of regional variation in response to spasmogens.

Table 2. Summary of spasmolytic potency (EC50) and relaxant activity (relaxation, % papaverine Emax) in guinea-pig isolated trachea contracted with different spasmogenic agonists.

Contractile agents	Spasmolytic	EC50 (95% CI) nM	Emax (% papaverine)	N
Histamine/Carbachol				
	RPL554	640 (177 – 2310)	100 ± 6	5
	Salbutamol	7 (1.2 – 44)	79 ± 25	5
	Ipratropium bromide	2.8 (0.6 – 15.0)	79 ± 15	5
	'Combivent'	16 (6.7 – 36.6)	100 ± 5	5
LTC_4/histamine/carbachol				
	RPL554	3393 (812-14180)	93 ± 9	6
	Salbutamol	3.2 (1 - 12)	67 ± 12*	6
	Ipratropium bromide	2.5 (1.0 - 6.1)	70± 19*	6
	'Combivent'	4.4 (1 – 19)	93 ± 9	6
KCl/carbachol				
	RPL554	15170 (4290-53660)	94 ± 25	10
	Salbutamol	9.9 (2.9 – 3.4)	43 ± 18*	5
	Ipratropium bromide	6.4 (1.5 – 27.1)	73± 15*	5
	'Combivent'	11.6 (3.9 – 34.4)	83 ± 10*	5
LTC_4/KCL/carbachol				
	RPL554	30111 (6406-141600)	97 ± 7	5
	Salbutamol	7.5 (1.8 – 30.7)	42 ± 18*	5
	Ipratropium bromide	7.8 (1.3 – 4.8)	56 ± 16*	5
	'Combivent'	41.8 (17.3 – 101)	53 ± 8*	5

EC50 and Emax parameter estimates were calculated for each curve by non-linear regression and the values represent the global geometric (95% confidence interval) and arithmetic mean (SD), respectively from N animals. * $P < 0.05$ compared with 100 % maximum relaxation (single sample t-test).

Table 3. Summary of spasmolytic potency (EC50) and relaxant activity (relaxation, % papaverine Emax) in guinea-pig isolated trachea contracted with different spasmogenic agonists.

Spasmolytic	EC50 (95% CI) nM	Emax (% papaverine)	N
Carbachol (10x EC50)†			
RPL554	584 (226 – 1507)	107 ± 12	10
Salbutamol	24.8 (15.4 – 39.9)	93 ± 10	7
Formoterol	0.15 (0.01 - 210)	93± 5	4
Cilostazol	1231 (225 - 6724)	98 ± 27	4
Cilostamide	738 (201 – 2708)	87 ± 21	4
Siguazodan	79 (9 – 709)	42 ± 12	3
Roflumilast	22.3 (6.3 – 78.6)	50 ± 15*	5
Histamine/ Carbachol			
RPL554	1617 (303 – 8614)	92 ± 11*¶	17
Salbutamol	24 (2 – 250)	51 ± 16*	7
Formoterol	6.1 (0.3 – 138)	54 ± 17*	6
Cilostamide	2046 (790 – 5303)	52 ± 21*	13
Siguazodan	18 (5 – 70)	49 ± 14*	5
Roflumilast	54 (4 – 650)	41 ± 12*	10
Cilostamide + Roflumilast (1:1)	27 (0.6 – 1162)	52 ± 24*	9
LTC_4/histamine/Carbachol			
RPL554	11080 (4234 – 29010)	99 ± 7	11
Cilostamide	428 (145 – 1262)	63 ± 10*	6
Roflumilast	19 (7 – 46)	63 ± 16	10
Cilostamide + Roflumilast (1:1)	54 (7 – 433)	63 ± 12*	6
Siguazodan	21 (7 – 63))	45 ± 21*	6
Siguazodan + Roflumilast (1:1)	119 (29 – 485)	50 ± 17*	5

EC50 and Emax parameter estimates were calculated for each curve by non-linear regression and the values represent the global geometric (95% confidence interval) and arithmetic mean (SD), respectively from N animals. * $P < 0.05$ compared with 100 % maximum relaxation (single sample t-test). ¶ $P < 0.05$ compared with other Emax values in this group (Benjamini-Hochberg procedure for multiple comparison). † No statistical analysis performed as these values serve to show in a qualitative fashion the magnitude of the relaxation response to each spasmolytic under this contractile condition.

Table 4. Calculation of interaction index (alpha) and the difference between observed and additive relaxation response (O-A) to combination glycopyrronium and RPL554.

Contractile agents	**Total concentration (nM)**	**Alpha (mean, SD)**	**O-A (% papaverine; mean, SD)**
LTC4/histamine/carbachol			
Glycopyrrolate/RPL554 (1:24) (N=6)	0.1	0.02 (0.02)	6 (5) ††
	0.3	0.02 (0.01)	15 (14) †
	1	0.03 (0.02)	23 (16)
	3	0.04 (0.04)	31 (16)
	10	0.10 (0.13)	35 (21)
Histamine/Carbachol			
Glycopyrrolate/RPL554 (1:24) (N = 5)			
	0.1	0.29 (0.36) †	5 (4) †
	0.3	0.17 (0.13)	13 (7) ††
	1	0.30 (0.13)	15 (6)
	3	0.41 (0.08)	18 (4)
Glycopyrrolate/RPL554 (1:226) (N = 5)			
	0.1	0.11 (0.21)	7 (5)
	0.3	0.07 (0.11)	12(8)
	1	0.08 (0.10)	20 (10)
	3	0.11 (0.07)	27 (13)
	10	0.19 (0.10)	34 (18)
	30	0.30 (0.15)	34 (17)
	100	0.48 (0.28)	22 (14)
	300	0.61 (0.34)	11 (7)
LTC4/KCL/carbachol			
Glycopyrrolate/RPL554 (1:24) (N = 6)			
	1	0.1 (0.2)	11 (6)
	3	0.04 (0.03)	14 (4)
	10	0.11 (0.09)	13 (4)
	30	0.51 (0.21)	17 (14)
	100	0.60 (0.37)	18 (18) †
	300	0.47 (0.30)	24 (17)
	1000	0.49 (0.30)	22 (15)
	3000	0.42 (0.14)	24 (8)

Interaction index (alpha) values less than unity are suggestive of a synergistic interaction (all alpha values. Delta (% O-A) represents the difference between the combined (observed: O) and the additive (A) response for each concentration pair. In both cases all values presented in this table were statistically different (alpha vs unity; %O-A vs zero) with P values adjusted using Benjamini-Hochberg procedure. Unlesss otherwise specified $P < 0.05$ following adjustment ($P > 0.05$ prior† and following ††adjustment).

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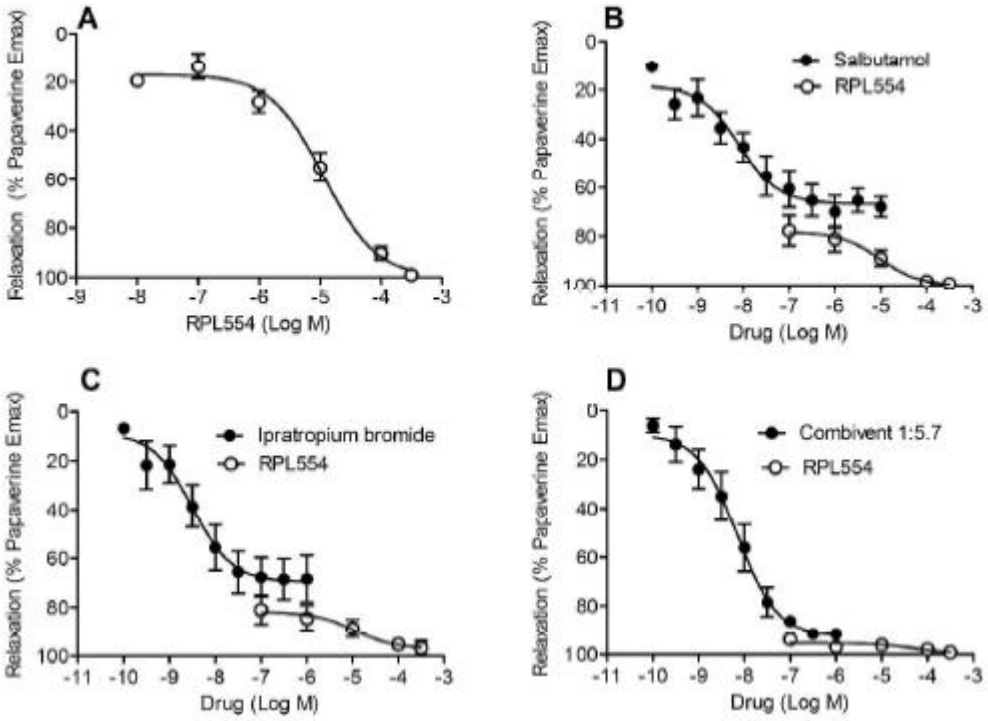

Figure 1. **RPL554 provides additional relaxation in combination with various spasmolytics**. Concentration-relaxation response curve to (A) RPL554 (open circles), (B) salbutamol, (C) ipratropium bromide and (D) 'Combivent (closed circles) in tissues contracted with LTC4 (100 nM), histamine (10 μM) and carbachol (10 μM). Additional relaxation response to RPL554 was also detected in tissues fully relaxed to the conventional bronchodilators. Data expressed as mean ± SEM, in tracheal tissue from N = 6 animals.

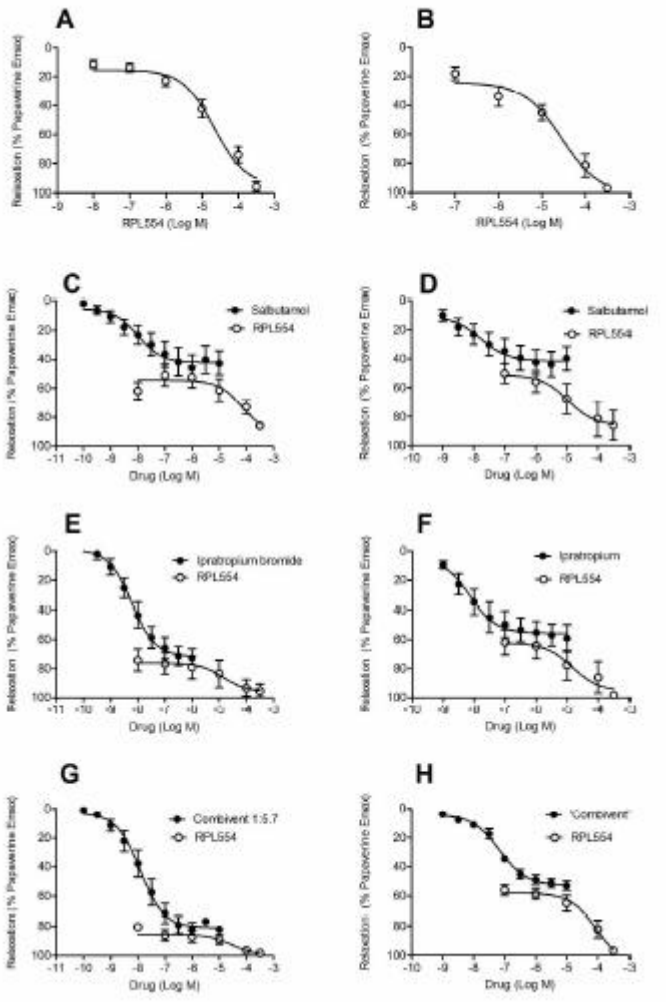

Figure 2. **RPL554 provides additional relaxation in combination with various spasmolytics**. Concentration-relaxation response curve to (A, B) RPL554 (open circles), (C,D) salbutamol, (E, F) ipratropium bromide and (G, H) 'Combivent' (closed circles) in tissues contracted with (A,C,E,G) KCL (40 mM) and carbachol (10 μM) or LTC4 (100 nM), KCL (40 mM) and carbachol (10 μM) (B,D,F,H). Additional relaxation response to RPL554 was also detected in tissues fully relaxed to the conventional bronchodilators. Data expressed as mean ± SEM, in tracheal tissue. Refer to Table 2 for N number. Data presented in lefthand and righthand panels of this figure was derived from different cohorts of animals.

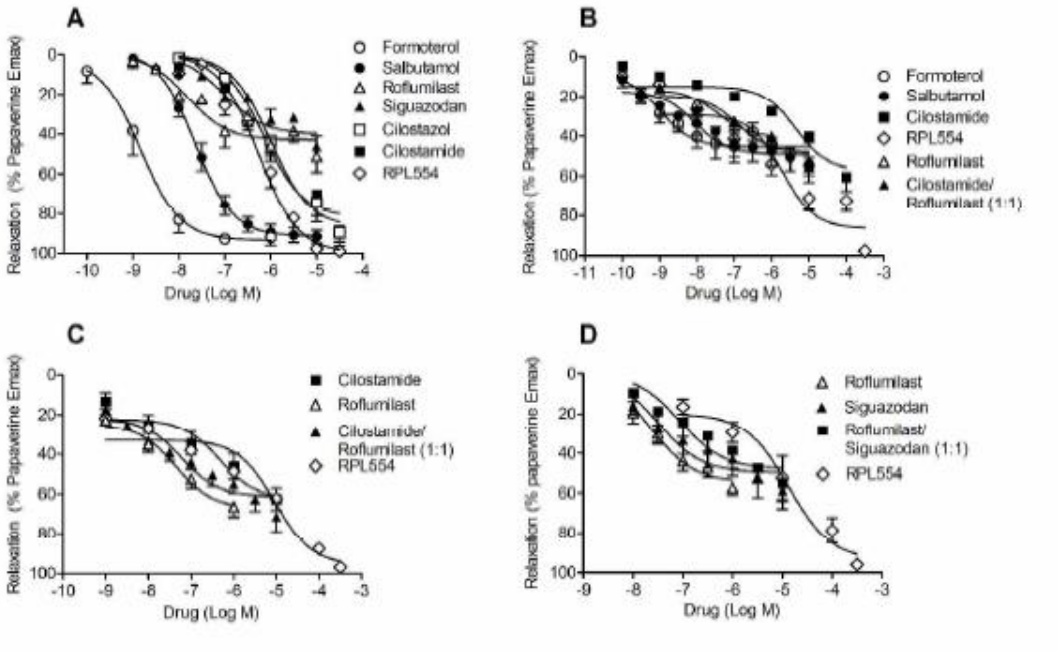

Figure 3. **A comparison of spasmolytic activity to various PDE inhibitors in guinea-pig isolated trachea.** Concentration-relaxation response curve to RPL554, PDE3 selective inhibitors (cilostazol, cilostamide and siguazodan), PDE4 selective inhibitor (roflumilast) and beta2-agonists (formoterol and salbutamol) in tissues contracted with (A) carbachol (10 x EC50); (B) histamine (10 μM) and carbachol (10 μM) and (C, D) LTC4 (100 nM), histamine (10 μM) and carbachol (10 μM). Data expressed as mean ± SEM, in tracheal tissue. Refer to Table 3 for N number. Data presented in each panel of this figure was derived from different cohorts of animals.

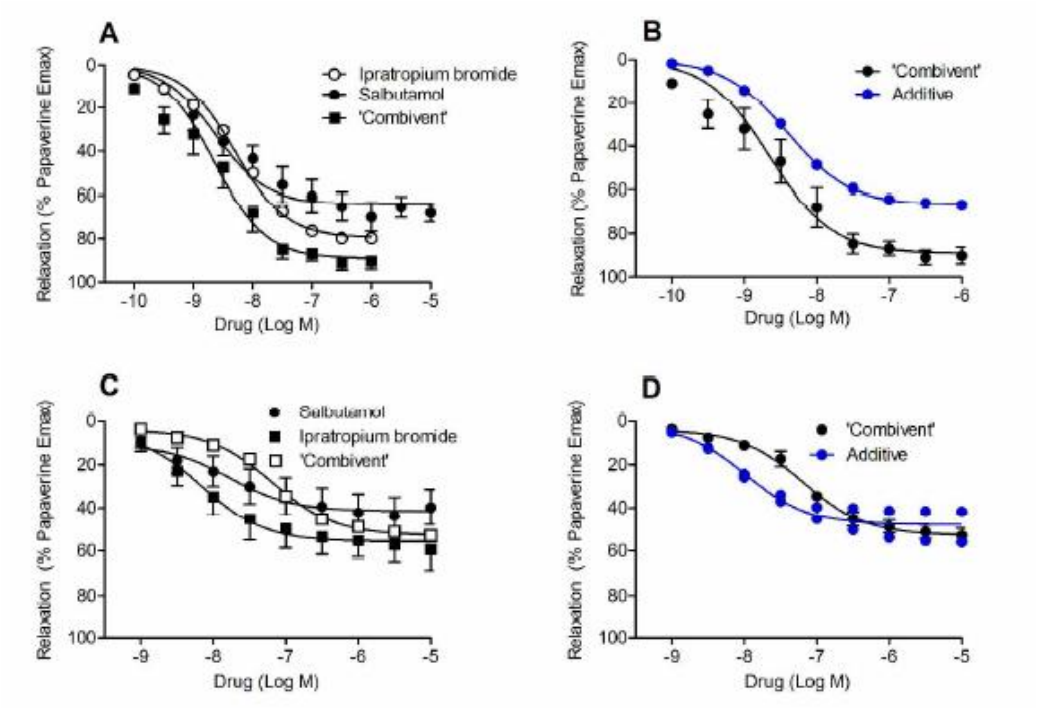

Figure 4. **Synergistic interaction between salbutamol and ipratropium bromide is inhibited by potassium chloride**. Concentration-relaxation response curve to salbutamol, ipratropium bromide and 'Combivent' in tissues contracted with (A) LTC4 (100 nM), histamine (10 μM) and carbachol (10 μM) and (B) the corresponding comparison of the concentration-response curve to the observed response to 'combivent' and the zero interaction relationship (additive). Data expressed as mean ± SEM, in tracheal tissue. Refer to Table 2 for N number. Concentration-relaxation response curve to (C) salbutamol, ipratropium bromide and 'Combivent' in tissues contracted with LTC4 (100 nM), KCL (40 mM) and carbachol (10 μM) and (D) comparison of the concentration-response curve to the observed response to 'combivent' and the zero interaction relationship (additive). Data expressed as mean ± SEM, in tracheal tissue. Refer to Table 2 for N number. Data represented in the upper and lower panels of this figure was obtained from different cohorts of animals.

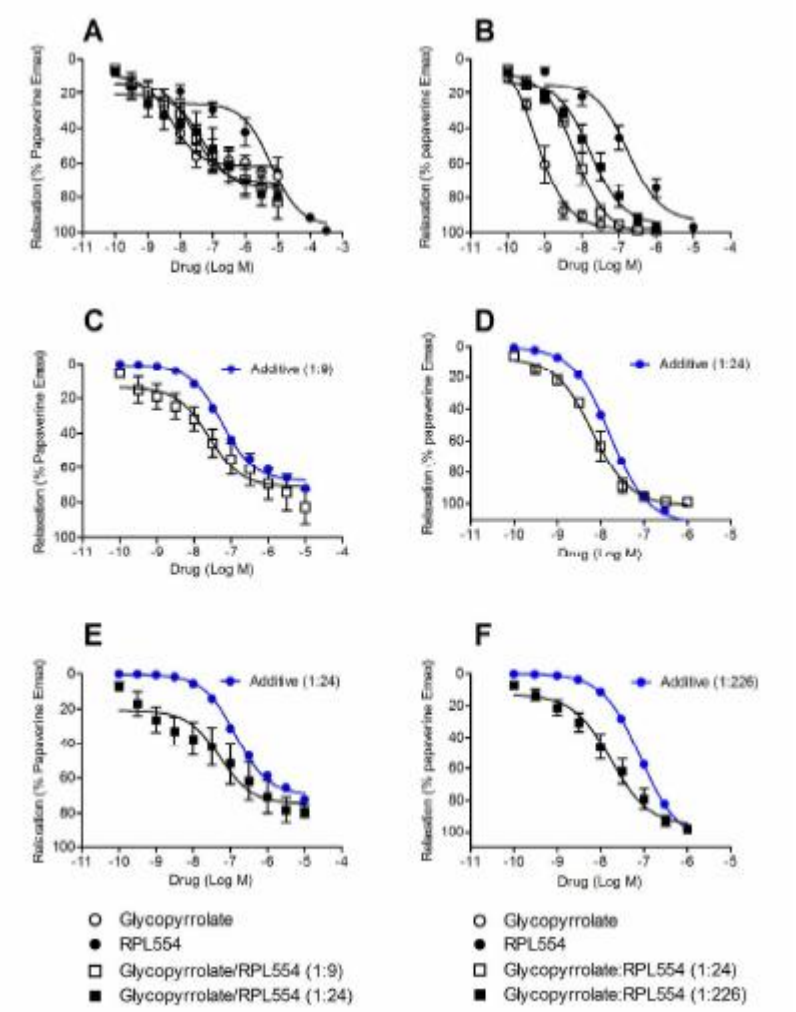

Figure 5. **Synergistic interaction between RPL554 and glycopyrrolate under various contractile conditions,** (A) Concentration-relaxation response curve to glycopyrrolate, RPL554 alone or in combination (1:9, 1:24) in tissues contracted with LTC4 (100 nM), histamine (10 μM) and carbachol (10 μM). Data expressed as mean ± SEM, in tracheal tissue from N = 5-6 animals. Comparison of the relaxation concentration-response curve for different combination ratios glycopyrrolate and RPL554 (C, 1:9 respectively and E, 1:24 respectively) and the zero interaction relationship (additive) for each concentration pair. Concentration-relaxation response curve to glycopyrrolate, RPL554 alone or in combination (1:24, 1:226) in tissues contracted with (B) histamine (10 μM) and carbachol (10 μM). Data expressed as mean ± SEM, in tracheal tissue from N = 5-7 animals. Comparison of the concentration-response curve to the observed response to the different combination ratios of glycopyrrolate and RPL554 (D, 1:249 respectively and E, 1:226 respectively) and the zero interaction relationship (additive) for each concentration. Data represented in the left and right panels of this figure was obtained from different cohorts of animals. There was evidence for a synergistic interaction between glycopyrrolate and RPL554 (see Table 4) as reflected by a significant interaction index (alpha) and difference between the observed and additive relaxation response (% mean (SD) difference).

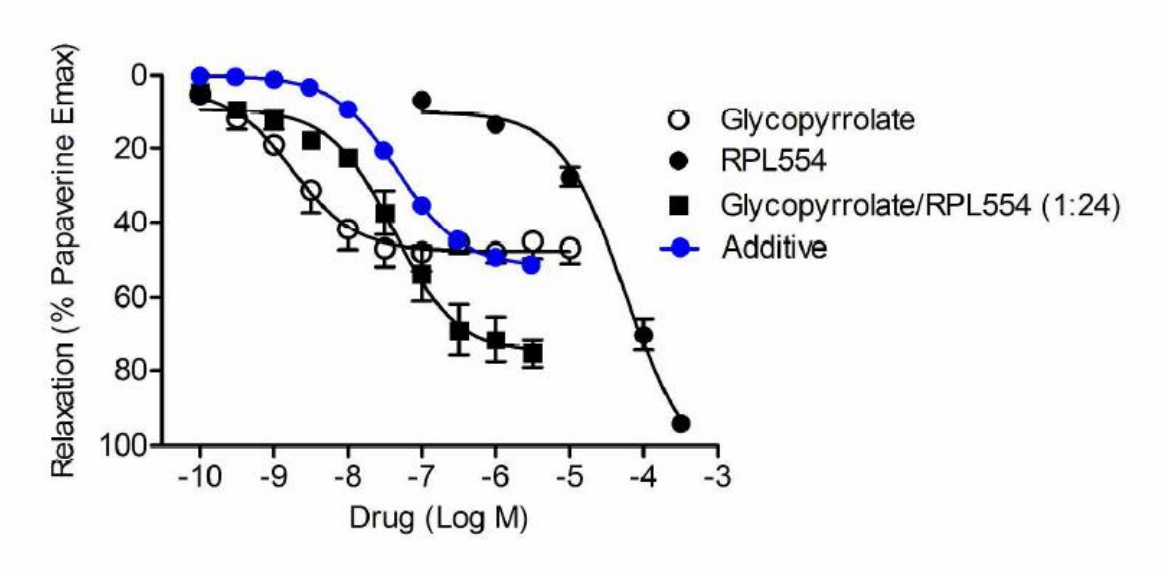

Figure 6. **Synergistic interaction between RPL554 and glycopyrrolate is not altered in the presence of potassium chloride.** Concentration-relaxation response curve to glycopyrrolate, RPL554 alone or in combination (1:24) in tissues contracted with LTC4 (100 nM), KCL (40 mM) and carbachol (10 μM). Data expressed as mean ± SEM, in tracheal tissue from N = 6 animals. For the analysis of synergistic interaction, the additive (zero interaction) concentration response curse (additive, blue curve) was calculated for each concentration pair. There was evidence for a synergistic interaction between glycopyrrolate and RPL554 (see Table 4) as reflected by a significant interaction index (alpha) and difference between the observed and additive relaxation response (% mean (SD) difference).

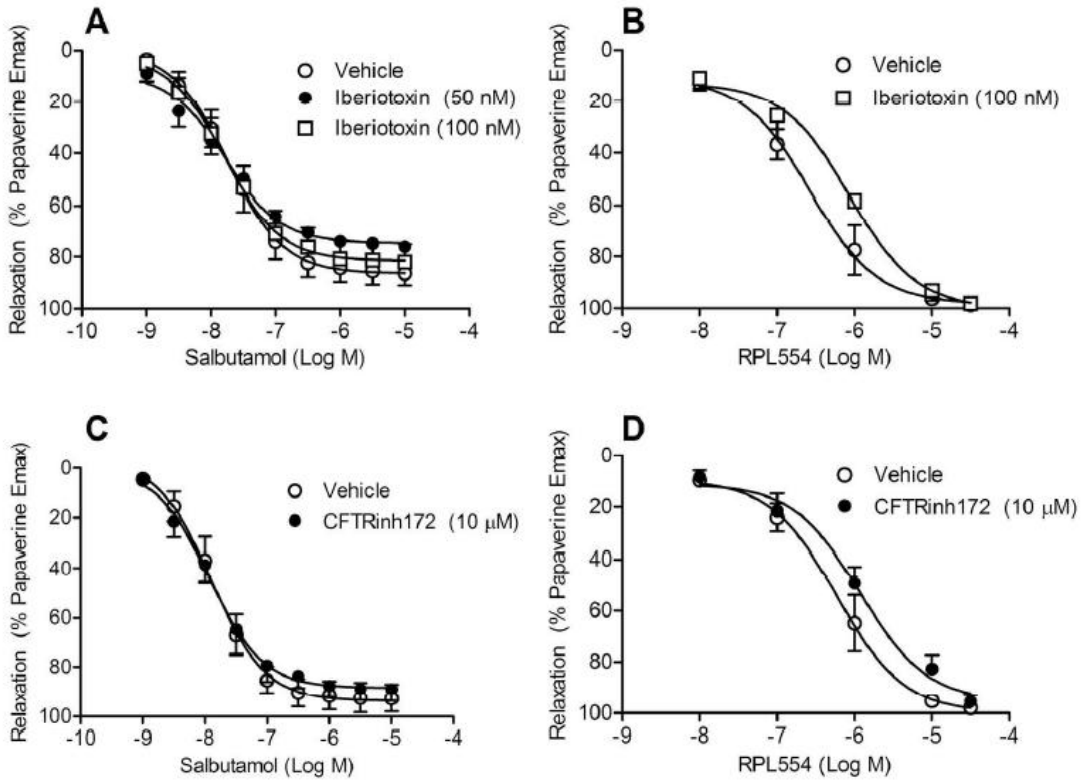

Figure 7. **Relaxation response to RPL554 is not mediated by calcium activated potassium channels or CFTR.** Concentration-relaxation response curve to salbutamol (A, C) or RPL554 (B, D) in the absence or presence of the calcium activated potassium channel blocker, iberiotoxin (A, B) or the cystic fibrosis transmembrane conductance regulator (CFTR) channel blocker, CFTRinh172 (C, D). Data expressed as mean ± SEM, in tracheal tissue from N = 6 and 5 animals per inhibitor group, respectively.

2. TABLES OF LINKS - TARGETS

A quick guide to completing the Tables of Links can be found HERE. (Please view as a **slideshow** to see the full animation), and a video can be found here.

Copy/Paste information into this table as shown in the example in grey shading below

TARGETS			
Nomenclature	**Target Id (insert after the standard URL below, no spaces)**	**Database page citation**	***Concise Guide to PHARMACOLOGY* citation**
PDE3A	http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=1298	Phosphodiesterases, 3',5'-cyclic nucleotide. Accessed on 30/03/2016. IUPHAR/BPS Guide to PHARMACOLOGY,http://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=260	Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M, Peters JA and Harmar AJ, CGTP Collaborators. (2013) The Concise Guide to PHARMACOLOGY 2013/14: G Protein-Coupled Receptors. *Br J Pharmacol.* 170: 1459–1581.
PDE3B	http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=1299	Phosphodiesterases, 3',5'-cyclic nucleotide. Accessed on 30/03/2016. IUPHAR/BPS Guide to PHARMACOLOGY,http://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=260.	Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M, Peters JA and Harmar AJ, CGTP Collaborators. (2013) The Concise Guide to PHARMACOLOGY 2013/14: G Protein-Coupled Receptors. *Br J Pharmacol.* 170: 1459–1581.
PDE4A	http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=1300	Phosphodiesterases, 3',5'-cyclic nucleotide. Accessed on 30/03/2016. IUPHAR/BPS Guide to PHARMACOLOGY,http://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=260.	Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M, Peters JA and Harmar AJ, CGTP Collaborators. (2013) The Concise Guide to PHARMACOLOGY 2013/14: G Protein-Coupled Receptors. *Br J Pharmacol.* 170: 1459–1581.

PDE4B	http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=1301	Phosphodiesterases, 3',5'-cyclic nucleotide. Accessed on 30/03/2016. IUPHAR/BPS Guide to PHARMACOLOGY,http://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=260.	Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M, Peters JA and Harmar AJ, CGTP Collaborators. (2013) The Concise Guide to PHARMACOLOGY 2013/14: G Protein-Coupled Receptors. *Br J Pharmacol.* 170: 1459–1581.
PDE4D	http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=1303	Phosphodiesterases, 3',5'-cyclic nucleotide. Accessed on 30/03/2016. IUPHAR/BPS Guide to PHARMACOLOGY,http://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=260.	Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M, Peters JA and Harmar AJ, CGTP Collaborators. (2013) The Concise Guide to PHARMACOLOGY 2013/14: G Protein-Coupled Receptors. *Br J Pharmacol.* 170: 1459–1581.

This table lists protein targets and ligands which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013a, Alexander *et al.*, 2013b).

3. TABLES OF LINKS - LIGANDS

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Copy/Paste information into this table as shown in the example in grey shading below

LIGANDS			
Ligand name	**Ligand Id (insert after the standard URL below, no spaces)**	**INN only**	**IUPAC Name**
cilostamide	http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=5167	cilostamide	N-cyclohexyl-N-methyl-4-[(2-oxo-1,2-dihydroquinolin-6-yl)oxy]butanamide
cilostazol	http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=7148	cilostazol	6-[4-(1-cyclohexyl-1H-1,2,3,4-tetrazol-5-yl)butoxy]-1,2,3,4-tetrahydroquinolin-2-one
roflumilast	http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=6962	rolfumilast	3-(CYCLOPROPYLMETHOXY)-N-(3,5-DICHLOROPYRIDIN-4-YL)-4-(DIFLUOROMETHOXY)BENZAMIDE
formoterol	http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=3465	formoterol	N-[2-hydroxy-5-[1-hydroxy-2-[1-(4-methoxyphenyl)propan-2-ylamino]ethyl]phenyl]formamide
ipratropium bromide	http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=325	ipratropium bromide	(8-methyl-8-propan-2-yl-8-azoniabicyclo[3.2.1]octan-3-yl) 3-hydroxy-2-phenylpropanoate
glycopyrrolate	http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=7459	glycopyrrolate	(1,1-dimethylpyrrolidin-1-ium-3-yl) 2-cyclopentyl-2-hydroxy-2-phenylacetate
salbutamol	http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=558	salbutamol	4-[2-(tert-butylamino)-1-hydroxyethyl]-2-(hydroxymethyl)phenol

Iberiotoxin	http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?tab=structure&ligandId=4218	iberiotoxin	pGlu-Phe-Thr-Asp-Val-Asp-Cys-Ser-Val-Ser-Lys-Glu-Cys-Trp-Ser-Val-Cys-Lys-Asp-Leu-Phe-Gly-Val-Asp-Arg-Gly-Lys-Cys-Met-Gly-Lys-Lys-Cys-Arg-Cys-Tyr-Gln
histamine	http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=1204	histamine	2-(3H-imidazol-4-yl)ethanamine
carbachol	**http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=298**	carbachol	2-carbamoyloxyethyl-trimethylazanium
indomethacin	http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=1909	indomethacin	2-[1-(4-chlorobenzoyl)-5-methoxy-2-methylindol-3-yl]acetic acid
leukotriene C4	http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=3354	LTC4	(5S,6R,7E,9E,11Z,14Z)-6-[(2R)-2-[[(4S)-4-amino-5-hydroxy-5-oxopentanoyl]amino]-3-(carboxymethylamino)-3-oxopropyl]sulfanyl-5-hydroxyicosa-7,9,11,14-tetraenoic acid

This table lists protein targets and ligands which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013a, Alexander *et al.*, 2013b).